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A liquid chromatographic method for the determination of promethazine enantiomers in human urine and serum using solid-phase extraction and fluorescence detection

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Abstract

A LC method was developed for the concurrent assay of *R*(+) and *S*(−) promethazine from human urine and serum. The method involves the use of solid-phase extraction for sample clean-up. Chromatographic resolution of the enantiomers was performed under isocratic conditions using a mobile phase of hexane–1,2-dichlorethane–absolute ethanol–trifluoroacetic acid (400:150:100:1, v/v/v/v) at a flow rate of 1 ml min^{-1} on a brush-type column KK-CARNU. The enantiomers were detected by fluorescence using an excitation wavelength of 250 nm and a 280 nm emission cutoff filter. Chlorpromazine was used as the internal standard for urine analysis. Standard addition was used for promethazine analysis from serum. Drug to internal standard ratios were linear from 0.25 to 10 μg ml^{-1} in urine. Serum levels were linear from 2 to 10 ng ml^{-1}.

Keywords: Enantiomers; LC; Promethazine; Serum; Urine

1. Introduction

Promethazine, 10-(2-dimethylamino-2-methylethyl)phenothiazine, is an antihistamine that has been used for the treatment of allergic symptoms due to drug hypersensitivity, and is indicated for use as an adjunct for both local and general anesthetics. Dosage forms are typically tablets, injectables and suppositories, and dosages range from 12.5 to 50 mg every 4–24 h. The half-life of promethazine is 7–15 h in urine and 6–8 h in serum. Urine and plasma concentrations for the racemic mixture in man range from 0.5–20 μg ml^{-1} to 2–20 ng ml^{-1}, respectively [1–3].

Promethazine has been typically analyzed as the racemate in biological fluids using liquid/liquid extraction followed by electrochemical analysis, UV-LC or fluorometric analysis [1–6]. Some qualitative reports of promethazine enantiomeric separations by LC in the reverse-phase mode have been described [7,8].

This paper describes a solid-phase extraction method using a mixed-phase extraction column along with the resolution of *R*(+) and *S*(−) promethazine enantiomers by normal-phase chromatography using the KK-CARNU brush-type LC column and fluorescence detection. The analysis time for both urine and serum was approximately 1 h and the chromatographic run time was approximately 16 min. The procedure was accurate and precise enough to support clinical studies for both urine and serum samples.

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2. Experimental

2.1. Apparatus

The chromatograph consisted of a pump (Model 2510, Varian, Walnut Creek, CA, USA), equipped with a 20 μl injector loop (Model 7125, Rheodyne, Cotati, CA, USA), a fluorescence detector with $\lambda^{ex} = 254$ nm and a 280 nm emission filter (Model Spectroflow 980, Kratos, Ramsey, NJ, USA), and an integrator (Model SP 4290, Spectra-Physics, San Jose, CA, USA). The column was a KK-CARNU (α-*R*-naphthyl)ethylurea, 4.6 mm i.d. × 10 cm, 5 μm particle size, (YMC, Wilmington, NC, USA).

2.2. Solvents and chemicals

Hexane (97%), phosphoric acid (concentrated), monobasic potassium phosphate (reagent grade), potassium hydroxide and 1,2-dichloroethane F.C.C. were all obtained from J.T. Baker (Phillipsburg, NJ, USA). Ethanol 200 proof was purchased from Midwest Grain Products Co. (Weston, MO, USA). Triethylamine HPLC grade was purchased from Fisher (Fairlawn, NJ, USA). Promethazine and trifluoroacetic acid were purchased from Sigma (St Louis, MO, USA). The *R*(+) and *S*(−) promethazine enantiomers used in this study were synthesized through crystallization using D and L dibenzoylated tartaric acid, and analyzed for purity using LC and polarimetry [7]. The purity of the individual promethazine enantiomers was determined to be > 99% (w/w). Blank urine was obtained from healthy male volunteers. Human serum was obtained from Instrumentation Laboratory (Fisher Scientific, Orangeburg, NY, USA, Cat # 3160-34).

2.3. Preparation of the mobile phase

The mobile phase was hexane–1,2-dichloroethane–ethanol–trifluoroacetic acid (400:150:100:1, v/v/v/v). The solution was filtered through a 0.45 μm nylon 66 membrane filter (Magna, Honeoye Fall, NY, USA) and vacuum degassed for 5 min prior to use. The mobile phase was delivered at a flow rate of 1.0 ml min^{-1}.

2.4. Preparation of stock solutions

A stock solution (500 μg ml^{-1}) of *R*(+) promethazine and *S*(−) promethazine (hydrochloride salts) calculated as promethazine base was prepared in double distilled deionized water and stored at 4 °C. A chlorpromazine hydrochloride internal standard (IS) stock solution (1 mg ml^{-1}) calculated as the free base was also prepared in double distilled deionized water and stored at 4 °C. Both solutions were prepared weekly.

2.5. Preparation of spiked human urine samples

Accurately measured aliquots (5, 10, 20, 100 and 200 μl) of the stock solution containing the promethazine enantiomers were added to individual 10 ml flasks followed by the addition of 100 μl of chlorpromazine IS solution. Human urine was then added to the required volume. The final concentrations of promethazine were 0.25, 0.5, 1, 5 and 10 μg ml per enantiomer, with an IS concentration of 10 μg ml^{-1}.

2.6. Preparation of spiked human serum samples

One ml of the promethazine stock solution was diluted with water to give a final concentration of 100 ng ml^{-1} per enantiomer. From the 100 ng ml^{-1} solution, accurately measured aliquots (60, 120, 150 and 300 μl) were added to 3 ml volumetric flasks and serum was added to the required volume. The final concentrations of promethazine were 2, 4, 5 and 10 ng ml^{-1} per enantiomer.

2.7. Assay method

A 3 ml certify (Varian, Harbor City, CA, 90710, USA, Cat # 1210-2051) solid-phase extraction cartridge was conditioned with two cartridge volumes, each of absolute methanol and 0.1 M potassium phosphate monobasic pH 6.0. Human urine (5 ml) or serum (3 ml) containing promethazine was diluted with 5 ml of double distilled deionized water and 1 ml of phosphate buffer and vortexed for 1 min. The sample containing IS was applied to the SPE cartridge at a vacuum of 2–3 in. Hg. The tubes containing the diluted samples were washed with 1 ml of phosphate buffer and applied to the sorbent before the SPE cartridge was allowed to dry. After the sample was completely applied, the SPE cartridge was allowed to dry

under full vacuum for 3–5 min. The SPE cartridge was then washed with 3 × 500 μl of 1% conc. HCl in methanol (v/v) and 2 × 500 μl of 1 M aqueous acetic acid. After each wash application, the cartridge was allowed to dry. The analytes were eluted from the cartridge with 6 × 1 ml of 2% ammonium hydroxide in absolute ethanol into 1.5 × 10 mm tubes.

The eluent was evaporated to dryness using a nitrogen stream with the tubes partially submerged in ambient (23 ± 2 °C) water. For urine samples, the residue was reconstituted with 1 ml of mobile phase and vortexed for 3 min. For serum samples, the residue was reconstituted with 300 μl of a solution containing 5 mg ml^{-1} each of *R*(+) and *S*(−) promethazine prepared in the mobile phase and vortexed for 3 min. (Note: Some SPE cartridge sorbent material remained on the test tube wall and did not reconstitute in the mobile phase.)

2.8. *Calculations*

Urine

The peak heights of each promethazine enantiomer and the IS were recorded for each sample. Five concentrations in the range 0.25–10 μg ml^{-1} of the enantiomers were used to construct the calibration curves. Linear regression analysis of the ratio of drug to internal standard peak-height (D/IS) versus promethazine enantiomer concentration in urine was performed. The concentration of each promethazine enantiomer in a given urine sample was calculated form the equation: D/IS = (slope)(promethazine conc.) + intercept.

Serum

The peak areas of each promethazine enantiomer were recorded for each sample. Four concentrations in the range of 2–10 ng ml^{-1} were used to construct the calibration curve for each enantiomer. Subtraction of each response from the standard addition amount was performed prior to linear regression analysis. Linear regression analysis of *R*(+) and *S*(−) promethazine was performed on adjusted peak areas versus promethazine enantiomer concentrations in serum. The concentration of each promethazine enantiomer in a given serum sample was calculated from the equation: peak area of D = (slope)(promethazine conc.) + intercept.

3. Results and discussion

The chemical structures of promethazine and chlorpromazine (IS) are shown in Fig. 1. Previous work in this laboratory developing enantiomeric separations on various types of chiral stationary phases and mobile phases showed that promethazine enantiomers were easily separated using the brush-type stationary phase found in the KK-CARNU column and a mobile phase of hexane–1,2-dichlorethane–absolute ethanol–trifluoroacetic acid (400:150:100:1, v/v/v/v) [9]. The stationary phase is an experimental phase being developed for market by the YMC Corporation. It is a naphthylurea-type phase which utilizes the π–π, H-bonding, steric hindrance and dipole interactions between promethazine and the stationary phase in order to separate the *R*(+) and *S*(−) enantiomers. This type of stationary phase is usually run in the normal-phase mode. Various solvents typically have different effects upon the chromatography. It was shown that 1,2-dichloroethane could be replaced with dichloromethane, but the absolute ethanol could not be replaced without significantly altering the chromatographic results. Chromatographic system suitability was based on the retention times, separation and capacity for the enantiomers and IS. Typical chromatograms for blank and spiked human urine containing *R*(+) and *S*(−) promethazine and IS are shown in Figs. 2(A) and 2(B). Typical chromatograms for blank and spiked serum containing *R*(+) and *S*(−) promethazine are shown in Figs. 3(C) and 3(D). These data indicated that the method was suitable for the analysis of promethazine enantiomers in human urine and serum. The order of elution for the enantiomers was determined to be *R*(+) then *S*(−) promethazine.

The recovery of *R*(+) and *S*(−) promethazine from urine and serum was studied using C18, C8, C2, cyclohexyl, phenyl, diol, cyanopropyl and Certify SPE bonded phases. Recoveries for both enantiomers from either

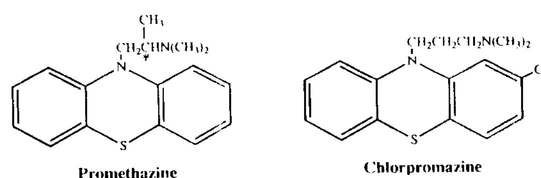

Fig. 1. Chemical structures of promethazine and chlorpromazine (IS).

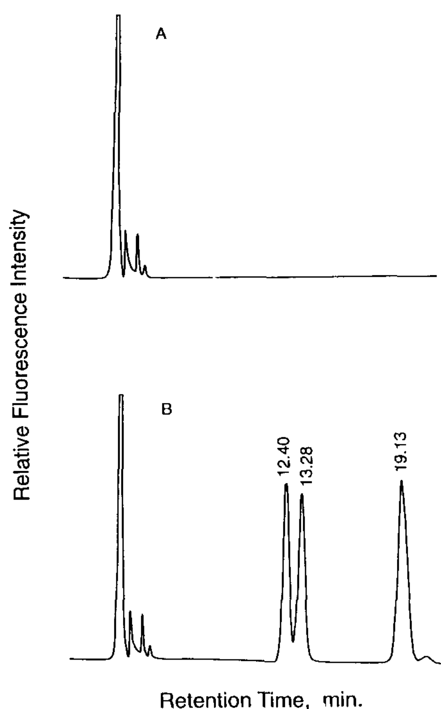

Fig. 2. Typical HPLC chromatograms of (A) blank human urine and (B) human urine with added $R(+)$ promethazine (12.40 min), $S(-)$ promethazine (13.28 min) and chlorpromazine IS (19.13 min).

biological fluid were similar and were highly dependent on the eluting solvent. Recoveries ranged from approximately 28% using a 1 cm^3 cyanopropyl cartridge to approximately 65% with a 1 cm^3 cyclohexyl cartridge. The 3 cm^3 cyanopropyl and 3 cm^3 cyclohexyl cartridges did not afford significantly higher recoveries for either enantiomer. Optimal recoveries were achieved on the 3 cm^3 Certify, which was specially designed for the extraction of trace analytes from urine by using a mixed bed of cation exchange, polar and non-polar sorbent materials. It was determined that the Certify cartridges worked well for both urine and serum samples in this study. However, these cartridges were sensitive to the conditioning environment used for sample preparation. Conditioning the Certify cartridge with water rather than phosphate buffer reduced the recoveries of both enantiomers by 33%. Elution of promethazine and chlorpromazine was also reduced by 33-90% when eluting with any other solvent besides an ammonia-ethanol mix. The ammonia was essential to the extraction as it was basic enough to increase the pH of the eluent to more than two units above the pK_a of the aliphatic amine (9.6) in the side chain, and was volatile enough to evaporate off under flowing nitrogen gas. Other solvents mixed with ammonia as potential eluents were acetone, methanol, propanol, acetonitrile, ethyl acetate and chloroform. Other potential internal standards investigated for this method were promazine, acepromazine and trifluoperazine; however, recoveries ranged from 63 to 67% with the ammonia-ethanol mix.

The absolute recoveries for $R(+)$ and $S(-)$ promethazine from the biological fluids were 93.2% $\pm$ 1.31 ($n = 8$) and 96.5% $\pm$ 3.19 ($n = 8$) from urine, and 93.1% $\pm$ 2.15 ($n = 8$) from serum, respectively. The absolute recovery of chlorpromazine was 79.1% $\pm$ 2.87 ($n = 8$) from urine and 78.5% $\pm$ 3.51 ($n = 8$) from serum. No interferences were observed in the blank human urine or serum at the retention times of $R(+)$ and $S(-)$ promethazine and chlorpromazine. Minimum detectable limits for each enantiomer in urine were 0.4 μg ml (S/N = 3).

The standard addition method was used to determine the amounts of $R(+)$ and $S(-)$ promethazine enantiomers in serum. The serum levels of both enantiomers were found to be below the detectable level of the assay reported above for urine levels. It was determined that adding 300 μl of a 5 mg ml^{-1} solution of each promethazine enantiomer to calibration stan-

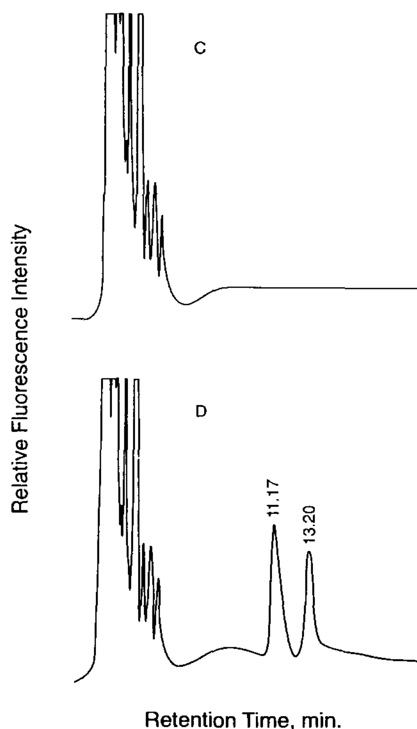

Fig. 3. Typical HPLC chromatograms of (C) blank serum and (D) serum with added $R(+)$ promethazine (11.17 min), and $S(-)$ promethazine (13.20 min).

Table 1
Analysis of spiked promethazine enantiomers in human urine and serum

Biological fluid	Analyte	Conc.		Error (%)	RSD (%)
		Added	Found		
Urine [a]	$R(+)$	3.00	2.92 ± 0.18	2.7	6.3
		8.00	8.58 ± 0.06	7.2	5.7
	$S(-)$	3.00	2.74 ± 0.01	8.7	3.6
		8.00	7.84 ± 0.22	2.0	4.8
Serum [b]	$R(+)$	3.33	3.33 ± 0.10	0.0	3.0
		7.50	7.80 ± 0.11	4.0	1.4
	$S(-)$	3.33	3.34 ± 0.03	0.3	0.9
		7.50	7.88 ± 0.26	1.2	2.6

[a] Concentrations in μg ml^{-1}.
[b] Concentrations in ng ml^{-1}.

dards and analytical samples after extraction and evaporation allowed the detection of small differences in peak areas after subtracting the response due to the added amount of promethazine. The peak area response for each calibration was adjusted by subtracting the peak area response from the standard addition response found at the y-intercept. Linear regression analysis of adjusted peak area versus promethazine concentration in the calibration standards was performed.

Linear regression analysis of $R(+)$ and $S(-)$ promethazine versus IS from urine gave r^2 values of 0.9993 and 0.9999, respectively ($n = 6$). Linear regression for the enantiomers in serum gave r^2 values of 0.9989 and 0.9872, respectively ($n = 6$). The accuracy and precision of both methods were determined using spiked samples of the enantiomers in urine and serum. The results shown in Table 1 indicate that the method has an accuracy in the range of 2–9% in urine and a precision in the range of 3.6–6.3%. The accuracy and precision data in serum were in the range 0–4% and 1–3%, respectively. The stability of the samples in the mobile phase was monitored for 24 h. No significant loss of detector response was observed for either promethazine enantiomer or internal standard.

4. Conclusions

Liquid chromatographic methods have been developed for $R(+)$ and $S(-)$ promethazine enantiomers in urine and serum. The methods should be suitable for the determination of promethazine enantiomers in either fluid at reported concentration levels, and should be applicable to clinical studies.

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